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STUDIES ON THE PECTIN PRESENT IN THE BARK OF WHITE WILLOW (*Salix alba* L.): FRACTIONATION AND ACIDIC DEPOLYMERIZATION OF THE WATER-SOLUBLE PECTIN

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ABSTRACT

An acidic polysaccharide complex, isolated by fractional precipitation of willow-bark pectin by Cetavlon, has been subjected to partial hydrolysis with acid. The structures of the acidic and neutral oligosaccharides were established using chromatographic methods, enzymic hydrolysis, and mass spectrometry of the products of methylation analysis. The following acidic oligomers were characterized: 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose, 4-*O*-(β-D-glucopyranosyluronic acid)-L-fucose, 2-*O*-(α-D-galactopyranosyluronic acid)-L-rhamnose, 4-*O*-(α-D-galactopyranosyluronic acid)-D-xylose, 6-*O*-(β-D-galactopyranosyluronic acid)-D-galactose, 4-*O*-(α-D-galactopyranosyluronic acid)-D-galactose, 4-*O*-(α-D-galactopyranosyluronic acid)-D-galacturonic acid, and the homologous tri-, tetra-, and penta-saccharides. The neutral oligosaccharides included 4-*O*-β-D-galactopyranosyl-D-galactose and the homologous tri- and tetra-saccharide, and 6-*O*-β-D-galactopyranosyl-D-galactose and the homologous β-(1→6)-linked trisaccharide.

INTRODUCTION

Pectic substances are present in primary cell wall and in the middle lamella of higher plants, especially during the early stages of cell differentiation[1]. They are most abundant in soft tissues (*e.g.*, sugar-beet pulp[2] and fruit peel[3,4]), but present in only small proportion in woody tissues[5]. In bark, the proportion of these substances is substantially higher than in the wood[6,7]. There have been few studies of their detailed structures[7–9].

We now report on willow-bark pectin.

RESULTS AND DISCUSSION

Previous studies on the holocellulose from the bark of twigs of young white-willow (*Salix alba* L.) showed that extraction with water at room temperature furnished crude pectic material containing D-galacturonic acid (degree of esterification,

27), D-galactose, and L-arabinose in the molar ratios 5.4:1:0.8, with low proportions of D-xylose, D-glucose, L-rhamnose, and L-fucose[10]. Electrophoretic studies indicated the pectin to be composed of a low proportion of neutral material, and of acidic components having different electrophoretic mobilities. The neutral material consisted mainly of a D-galactan, the structure of which has been described[10]. The current studies were on the co-existing, complex, acidic polysaccharides.

Fractional precipitation by Cetavlon gave an electrophoretically homogeneous heteropolysaccharide complex containing D-galacturonic acid (~78%, degree of esterification 20), D-galactose, and L-arabinose, and lower proportions of L-rhamnose, D-xylose, and L-fucose.

The components in a partial, acid hydrolysate of the polymer were separated, using an anion-exchange resin, into neutral and acidic oligosaccharides. The acidic oligomers were of two types: (*1*) α-(1→4)-linked oligosaccharides of D-galacturonic acid, up to a pentasaccharide[10]; and (*2*) acidic components including the following aldobiouronic acids: β-D-Glc*p*A-(1→6)-D-Gal*p*, β-D-Glc*p*A-(1→4)-L-Fuc*p*, α-D-Gal*p*A-(1→2)-L-Rha*p*, α-D-Gal*p*A-(1→4)-D-Xyl*p*, α-D-Gal*p*A-(1→4)-D-Gal*p*, β-D-Gal*p*A-(1→6)-D-Gal*p*.

The nature of the various glycosiduronic linkages was determined by mass spectrometry. The anomeric configurations were established by the use of β-D-glucuronidase, or of α- and β-D-galactosidases on the carboxyl-reduced saccharides.

Methylation (Hakomori[11]) of the aldobiouronic acids led to β-elimination and the formation of 4,5-unsaturated uronic acid derivatives. These derivatives were identified by mass spectrometry, as they yielded characteristic ions with *m/e* values 32 mass-units less than those of the parent disaccharides, and also by the appearance of the aH_1 ions at *m/e* 144 and abH_1 ions at *m/e* 292, 262, and 248 (where *b* = hexose, deoxyhexose, or pentose) which are typical of this type of compound[12].

The neutral oligosaccharides were fractionated on Sephadex G-10, and the fraction containing a mixture of di-, up to penta-, saccharides was further purified by p.c. The following oligosaccharides were identified: 4-*O*-β-D-galactopyranosyl-D-galactose and the homologous tri- and tetrasaccharide, and 6-*O*-β-D-galactopyranosyl-D-galactose and the homologous trisaccharide. Oligosaccharides containing L-arabinose residues are under study.

The results show that many of the structural features in other pectins[4,9,13,14] are also present in willow-bark pectin. The most frequently occurring aldobiouronic acid residue is 2-*O*-(α-D-galactopyranosyluronic acid)-L-rhamnose, which derives from the rhamnogalacturonan chain of the pectinic acid. The role this unit plays in forming the characteristic shape of rhamnogalacturonan and in its distribution have been elucidated[4,14]. The position of the other aldobiouronic acid residues is unknown. They are probably present either as terminal residues or in the outer chains of the pectinic acid. The isolation of 6-*O*-(β-D-galactopyranosyluronic acid)-D-galactose from a partial hydrolysate of willow-bark pectin apparently provides the first evidence of the existence of this structural analogue of the frequently occurring unit 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose.

The neutral oligosaccharides probably originate from the polymeric chains attached to a rhamnogalacturonan core. The β-(1→4)- and β-(1→6)-linked D-galactose residues together with L-arabinose residues may be structural units of the polysaccharide chains. However, it is not known whether these polysaccharides are heteropolymers or mixtures of homopolymers having various glycosidic bonds. The neutral D-galactan isolated from the pectic material contained[10] β-(1→4)- and β-(1→6)-links. It may therefore be assumed that some of the D-galactose-containing outer chains were biosynthesised in the same way, so that a combination of β-(1→4) and β-(1→6) bonds cannot be excluded.

EXPERIMENTAL

General. — P.c. was performed by the descending method on Whatman No. 1 and No. 3MM papers, using: *A* 18:3:1:4 ethyl acetate–acetic acid–formic acid–water, *B* 5:5:1:3 ethyl acetate–pyridine–acetic acid–water, *C* 18:7:8 ethyl acetate–acetic acid–water, *D* 10:3:3 1-butanol–pyridine–water, and *E* 10:4:3 ethyl acetate–pyridine–water. The mobility (R_{GALA}) of the acidic sugars is expressed relative to that of D-galacturonic acid, and that (R_{ARA}) of neutral sugars is related to L-arabinose. T.l.c. was carried out on Silica gel G with 10:1 chloroform–acetone or 3:1 benzene–acetone.

Polysaccharides were subjected to high-voltage paper electrophoresis on glass-fibre sheets (Whatman GF 81) at 40 V/cm in pyridine–acetate buffer (pH 6.5, containing 10% of pyridine and 0.3% of acetic acid). Polymers were detected with *p*-anisidine[15]. Reducing sugars were run on Whatman No. 1 paper, in 50mM sodium tetraborate (pH 9.2) and 0.1M ammonium formate (pH 3.8) buffers, at 25 V/cm and detected with aniline hydrogen phthalate[16]. Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter for aqueous solutions at ~22°.

Oligosaccharides were methylated by the Hakomori method[11]. The methylated derivatives were purified by t.l.c. before analysis by mass spectrometry. Mass spectra were obtained with an A.E.I. MS-902S spectrometer at an ionising potential of 70 eV. The inlet temperature was 50–60° and that of the ionising chamber 135–145°. The symbols A–J, employed by Kochetkov *et al.*[17], are used to denote the fragment ions, and *a* and *b* to designate the monosaccharide residues[18].

The acidic oligosaccharides were hydrolyzed in 90% formic acid at 100° for 18 h, and neutral oligomers for 4 h. Uronic acid content was determined by the carbazole method[19].

Isolation and fractionation of willow-bark pectin. — Sawdust (600 g) prepared from the bark of twigs of young white-willow (*Salix alba* L.) was extracted[10] with water at room temperature to give a crude pectic material (61 g). Paper electrophoresis of this polymeric mixture suggested a composition of a small proportion of neutral polymers (hydrolysis of which gave mainly D-galactose with traces of L-arabinose, D-xylose, and D-glucose), and a major and three minor acidic polysaccharides.

Aqueous, 5% cetyltrimethylammonium bromide (1.5 l) was added dropwise, with stirring, to a solution of pectin (25 g) in water (2.5 l). The resulting precipitate

was collected by centrifugation, washed thoroughly with water, and dissolved in 5% acetic acid, and the solution was poured into ethanol (4 l). The precipitated polysaccharide mixture (20 g) was collected, and dissolved in water, and the solution was freeze-dried (Found: uronic acid residues, 78%; degree of esterification, 20). Hydrolysis gave mainly D-galacturonic acid, D-galactose, and L-arabinose, together with small amounts of L-rhamnose, D-xylose, and L-fucose. Paper electrophoresis revealed the presence of a homogeneous, acidic polysaccharide fraction and of neutral polymers, although in considerably diminished amount.

Partial, acid hydrolysis of the acidic heteropolymer. — The heteropolymer (25 g) was heated with 0.5M sulphuric acid (1 litre) for 6 h at 100°. After cooling, the degraded polysaccharide was removed by centrifugation, and further polysaccharide was precipitated with ethanol (4 vol.). The combined, degraded polysaccharides were rehydrolysed until hydrolysis of the residual, degraded polymeric material (7.3 g) gave D-galacturonic acid and only traces of neutral sugars. The supernatant solutions were neutralized with barium carbonate, and the precipitated barium salts were removed by centrifugation and washed thoroughly with water adjusted to pH 4 with sulphuric acid. The combined centrifugate and washings were concentrated, passed through a column of Ionenaustauscher I (H^+) resin to remove barium ions, neutralised by shaking with Ionenaustauscher II (HO^-) resin, and concentrated.

The syrupy residue (15.2 g) was diluted with water and adsorbed batchwise on to a column (4.3 × 50 cm) of Dowex-1 x8 ($HCOO^-$) resin (100–200 mesh). Elution of the column with water removed neutral sugars (4 g) and elution with a gradient of 0.15 → 0.5M formic acid, and with 0.5M and 0.75M formic acid gave fractions containing D-galacturonic acid and acidic oligosaccharides. Further separations by preparative p.c. afforded the following chromatographically and electrophoretically homogeneous oligosaccharides.

6-*O*-(β-D-Glucopyranosyluronic acid)-D-galactose (118 mg), $[\alpha]_D$ −5° (*c* 0.83), R_{GALA} 0.18 (solvent *A*), gave D-glucuronic acid, D-glucurono-6,3-lactone, and D-galactose on acid hydrolysis. M.s. of a permethylated sample gave the ions aA_1, aA_2, aA_3 at *m/e* 233, 201, 169*; abJ_1*, bA_1, bA_2* at *m/e* 279, 219, 187, and the ion baD_1 at *m/e* 367 which proved[18] the presence of a (1→6)-linkage between the glycosyluronic residue and the reducing sugar unit. A solution of the disaccharide (1 mg) in 50mM sodium acetate buffer (1.3 ml, pH 4.5) was treated with β-D-glucuronidase (Calbiochem) for 24 h at 38°. After deionisation with a mixed-bed ion exchanger (Ionenaustauscher V), p.c. showed the presence of D-galactose and D-glucuronic acid.

4-*O*-(β-D-Glucopyranosyluronic acid)-L-fucose (13 mg), $[\alpha]_D$ −76° (*c* 0.8), R_{GALA} 0.50 and 1.03 (solvents *A* and *B*), gave D-glucuronic acid, D-glucurono-6,3-lactone, and L-fucose on acid hydrolysis. The ions at *m/e* 394, 319, 189 (baB_1, baF_1^2, bA_1), and mainly at 161 (bB_3) in the mass spectrum of the permethylated derivative proved[18] the glycosidic bond to be (1→4). Treatment of the disaccharide with β-D-glucuronidase yielded D-glucuronic acid and L-fucose.

*These ions are omitted in the further text.

2-*O*-(α-D-Galactopyranosyluronic acid)-L-rhamnose (205 mg), $[\alpha]_D$ +101° (*c* 1.0), R_{GALA} 0.78 (solvent *A*), gave D-galacturonic acid and L-rhamnose on acid hydrolysis. Peaks in the mass spectrum of the permethylated disaccharide at *m/e* 394, 319, and 189, and the absence of the peak at *m/e* 161, confirmed[18] the linkage to be (1→2). The aldobiouronic acid was converted into the corresponding methyl glycoside methyl ester (3% methanolic hydrogen chloride, 6 h, 100°) and reduced with lithium aluminium hydride in tetrahydrofuran[20]. When a solution of the reduced disaccharide (1 mg) in a sodium acetate buffer (1.0 ml, pH 4.8) was treated with α-D-galactosidase for 20 h at 37°, D-galactose and L-rhamnose were formed (p.c.).

4-*O*-(α-D-Galactopyranosyluronic acid)-D-xylose (10 mg), $[\alpha]_D$ +75° (*c* 0.75), R_{GALA} 0.40 (solvent *A*), gave D-galacturonic acid and D-xylose on acid hydrolysis. Peaks in the mass spectrum of the permethylated aldobiouronic acid at *m/e* 394, 319, 175, and 161 proved[18] the presence of a (1→4)-linkage. Treatment of the reduced disaccharide with α-D-galactosidase yielded D-galactose and D-xylose (p.c.).

4-*O*-(α-D-Galactopyranosyluronic acid)-D-galactose (17 mg), $[\alpha]_D$ +119° (*c* 1.0), R_{GALA} 0.29 (solvent *A*), gave D-galacturonic acid and D-galactose on acid hydrolysis. The ions at *m/e* 394, 319, 219, and at 161 in the mass spectrum of the permethylated derivative proved[18] the linkage to be (1→4). The reduced dimer gave D-galactose on treatment with α-D-galactosidase.

6-*O*-(β-D-Galactopyranosyluronic acid)-D-galactose (35 mg), $[\alpha]_D$ +39° (*c* 1.45), R_{GALA} 0.50 and 0.92 (solvents *A* and *B*), gave D-galacturonic acid and D-galactose on acid hydrolysis. The ions at *m/e* 219 and 367 in the mass spectrum of the permethylated derivative confirmed[18] the glycosidic bond to be (1→6). When a solution of the reduced dimer (1 mg) in sodium acetate buffer (1.5 ml, pH 7.2) was treated with β-D-galactosidase (Sigma) for 24 h at 37°, D-galactose was formed (p.c.).

During mass spectrometry of the permethylated aldobiouronic acids, the ions baD_1, baB_1, baF_1^2, and the ions of the C series with the values lower by 32 mass-units, together with the most prominent ions of the H series (aH_1 and abH_1 at *m/e* 144 and at 292, 262, and 248, where *b* = hexose, deoxyhexose, or pentose) could be detected. This indicated the presence of 4,5-unsaturated uronic acid derivatives[12].

4-*O*-(α-D-Galactopyranosyluronic acid)-D-galacturonic acid (520 mg), $[\alpha]_D$ +104° (*c* 1), R_{GALA} 0.17 and 0.52 (solvents *A* and *C*), was chromatographically and electrophoretically indistinguishable from galacturonobiose isolated after treatment of pectin with endopolygalacturonase[10], and gave D-galacturonic acid on acid hydrolysis. The homologous trisaccharide (80 mg, R_{GALA} 0.28), tetrasaccharide (10 mg, R_{GALA} 0.14), and pentasaccharide (traces, R_{GALA} 0.05) were isolated, and identified on the basis of chromatographic mobility in comparison with authentic standards (p.c., solvent *C*).

A solution of the neutral carbohydrate mixture (4 g) in water was added batchwise to a column (2.6 × 110 cm) of Sephadex G-10. Elution of the column with water gave (*1*) void-volume oligomers (464 mg), (*2*) a mixture of di- to pentasaccharides (353 mg) (fractions *1* and *2* gave mainly D-galactose and L-arabinose on

acid hydrolysis), (*3*) a mixture of monosaccharides (3.16 g) containing mainly D-galactose and L-arabinose.

When fraction *2* was subjected to preparative p.c., the following chromatographically and electrophoretically homogeneous oligosaccharides were obtained.

4-*O*-β-D-Galactopyranosyl-D-galactose (80 mg), $[\alpha]_D$ +62° (*c* 1), R_{ARA} 0.27 and 0.49 (solvents *D* and *E*), yielded D-galactose on acid hydrolysis. Peaks in the mass spectrum of the permethylated disaccharide at *m/e* 380, 305, 219, and mainly at 161 proved[21] the linkage to be (1→4), and the β configuration was established as described above. The homologous trisaccharide (25 mg, R_{ARA} 0.12, 0.3) and tetrasaccharide (10 mg, R_{ARA} 0.05, 0.15) were also isolated.

6-*O*-β-D-Galactopyranosyl-D-galactose (20 mg), $[\alpha]_D$ +28° (*c* 1), R_{ARA} 0.23 and 0.52 (solvents *D* and *E*), gave D-galactose on acid hydrolysis. The ions at *m/e* 219 and 353 in the mass spectrum of the permethylated derivative confirmed[21] the presence of a (1→6)-linkage, and the β configuration was established as described above. The homologous trisaccharide (23 mg), R_{ARA} 0.14 and 0.31 (solvents *D* and *E*), was also isolated.

REFERENCES

1 J. P. THORNBER AND D. H. NORTHCOTE, *Biochem. J.*, 81 (1961) 449, 455.
2 V. ZITKO AND C. T. BISHOP, *Can. J. Chem.*, 43 (1965) 3206.
3 A. J. BARRETT AND D. H. NORTHCOTE, *Biochem. J.*, 94 (1965) 617.
4 G. O. ASPINALL, J. W. T. CRAIG, AND J. L. WHYTE, *Carbohyd. Res.*, 7 (1968) 442.
5 E. ANDERSON, *J. Biol. Chem.*, 165 (1946) 233.
6 K. V. RAMALINGAM AND T. E. TIMELL, *Sv. Papperstidn.*, 67 (1964) 512.
7 T. J. PAINTER AND C. B. PURVES, *Tappi*, 43 (1960) 729.
8 S. S. BHATTACHARJEE AND T. E. TIMELL, *Can. J. Chem.*, 43 (1965) 758.
9 N. BANERJI AND N. S. THOMPSON, *Cellulose Chem. Technol.*, 2 (1968) 655.
10 R. TOMAN, Š. KARÁCSONYI, AND V. KOVÁČIK, *Carbohyd. Res.*, 25 (1972) 371.
11 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205.
12 V. KOVÁČIK AND P. KOVÁČ, *Anal. Biochem.*, 64 (1975) 45.
13 G. O. ASPINALL, I. W. COTTRELL, S. V. EGAN, I. M. MORRISON, AND J. N. C. WHYTE, *J. Chem. Soc., C*, (1967) 1071.
14 K. W. TALMADGE, K. KEEGSTRA, W. D. BAUER, AND P. ALBERSHEIM, *Plant. Physiol.*, 51 (1973) 158.
15 D. H. NORTHCOTE, *Methods Carbohyd. Chem.*, 5 (1965) 49.
16 S. M. PARTRIDGE, *Nature (London)*, 164 (1949) 443.
17 N. K. KOCHETKOV AND O. S. CHIZHOV, *Advan. Carbohyd. Chem.*, 21 (1966) 39.
18 V. KOVÁČIK, Š. BAUER, J. ROSÍK, AND P. KOVÁČ, *Carbohyd. Res.*, 8 (1968) 282.
19 T. BITTER AND H. M. MUIR, *Anal. Biochem.*, 4 (1962) 330.
20 M. ABDEL-AKHER AND F. SMITH, *Nature (London)*, 166 (1950) 1037.
21 O. S. CHIZHOV, L. A. POLJAKOVA, AND N. K. KOCHETKOV, *Dokl. Akad. Nauk SSSR*, 158 (1964) 685.